ウェブサイト公開用

（様式10）
（海外特別研究員事業）
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海外特別研究員最終報告書

独立行政法人日本学術振興会 理事長　殿

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受付番号　201960397
氏　　名　吉原 正仁

（氏名は必ず自署すること）

海外特別研究員としての派遣期間を終了しましたので、下記のとおり報告いたします。
なお、下記及び別紙記載の内容については相違ありません。

記

1. 用務地（派遣先国名）用務地：　ストックホルム　（国名：　スウェーデン　）

2. 研究課題名（和文）※研究課題名は申請時のものと違わないように記載すること。
再生医療を目指した新規リプログラミング因子によるヒト初期胚様幹細胞の作製

3. 派遣期間：平成・[令和]　元　年　8　月　1　日　～　令和　3　年　7　月　31　日

4. 受入機関名及び部局名
受入機関名：　Karolinska Institutet
部局名：　Department of Biosciences and Nutrition

5. 所期の目的の遂行状況及び成果…書式任意 **書式任意（A4 判相当 3 ページ以上、英語で記入も可）**

（研究・調査実施状況及びその成果の発表・関係学会への参加状況等）

（注）「6. 研究発表」以降については様式 10－別紙 1～4 に記入の上、併せて提出すること。

I have been interested in regenerative medicine using induced pluripotent stem cells (iPSCs) and worked on related projects as a clinician and researcher when I was working in Japan. However, I realized that there are still some problems to be solved, such as low efficiency of reprogramming, heterogeneity of cells, and genomic instability (Yoshihara et al. *Stem Cell Rev Rep*. 2017; Yoshihara et al. *Cell Rep*. 2017). During the fellowship period, first I studied the role of genes associated with early human embryonic development for application to novel reprogramming technologies. By targeting these promoter regions or genes, I succeeded in i) high fidelity reprogramming with CRISPR activation, and ii) transcriptional reprogramming of human embryonic stem cells to blastomere-like cells. Furthermore, using the iPSC-derived neuroepithelial stem cells and neural progenitor cell lines, I revealed transcriptomic changes during neuronal differentiation. Finally, I investigated the human tissue samples by targeted RNA-seq and single-cell RNA-seq for clinical applications (*equally contributed, #corresponding author).

I. Investigation of the role of DUX4 during early human embryonic development

In the oocyte-to-embryo transition, fertilized oocyte undergoes final maturation and embryonic genome is gradually activated during the first three cell divisions. In human, how this transition is coordinated and which factors drive this process are still largely unknown. Our group previously identified DUX4 as an early regulator of human embryonic genome activation (EGA; Töhönen et al. *bioRxiv*. 2017).

Here I studied the role of DUX4 during human early development by transcriptome and epigenome analysis. Although DUX4 knockdown (siDUX4) human zygotes did not arrest during the two-day culture after microinjection, significant number of maternal transcripts which should be degraded during the oocyte-to-embryo transition were retained in the siDUX4 zygotes. When DUX4 was overexpressed in human embryonic stem cells (hESCs), I discovered that 23 out of the 32 genes typically activated during the transition from oocyte to 4-cell stage were significantly upregulated (**Figure 1**). Interestingly, regions that gained chromatin accessibility by DUX4 overexpression were overrepresented with ERVL-MaLR retrotransposon long terminal repeat (LTR) elements, which were significantly enriched with DUX4 binding sites. These observations indicate that DUX4 modulates the degradation of maternal transcripts and works as a powerful activator of early embryonic genes and LTR elements during the oocyte-to-embryo transition. These results provide us better understanding of regulatory mechanism during the first days of human embryonic development and new insights into the stem cell technologies (Vuoristo, Hydén-Granskog, Yoshihara et al. *bioRxiv*. 2020).

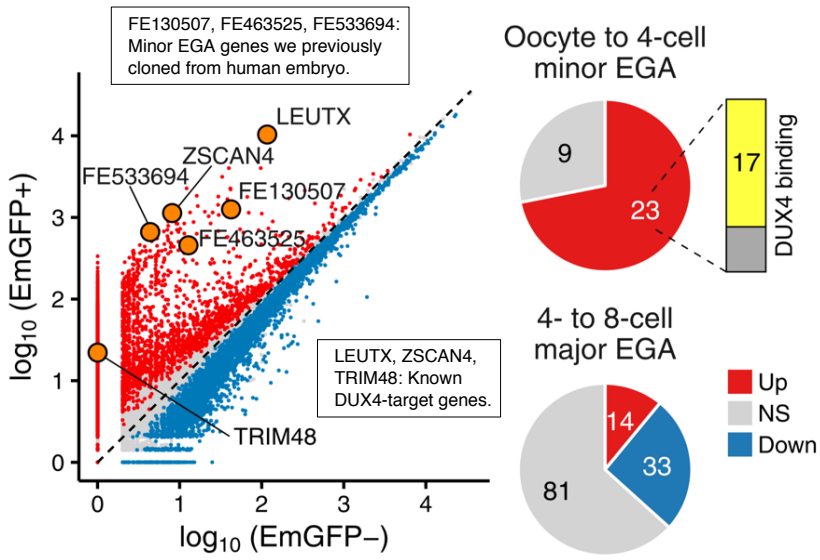

Figure 1. Differentially expressed genes by DUX4 overexpression in human embryonic stem cells (red: upregulated, blue: downregulated, gray: not significant). Pie charts represent the proportion of these genes in minor and major embryonic genome activation (EGA) genes.

II. More direct and synchronized reprogramming by CRISPR activators

Our group first reported that human skin fibroblasts can be reprogrammed into iPSCs using CRISPR/Cas9-based gene activation system (CRISPRa) targeting endogenous *OCT4*, *SOX2*, *KLF4*, *MYC*, and *LIN28A* (OSKML) promoters in addition with Alu-motif, which is enriched upstream of genes involved in embryonic genome activation (EEA-motif; Weltner et al. *Nat Commun.* 2018).

Here we succeeded in reprogramming human lymphoblastoid cell lines (LCLs) into iPSCs using CRISPRa targeting endogenous OSKML promoters and EEA-motif, albeit with the low reprogramming efficiency. However, the efficiency was drastically improved when LCLs were transfected in addition with the guides targeting specific region (confidential), which is known to promote pluripotency. We found cell colonies during the reprogramming process were larger and more homogeneous with our method than those with the conventional transgenic method. Furthermore, by single-cell transcriptome analysis, I revealed that our method reprogrammed the cells towards the pluripotent state more directly and quickly, whereas the iPSCs generated with conventional transgenic method showed higher heterogeneity including differentiating into other cell fates during the reprogramming process (**Figure 2**). I further confirmed that iPSCs at late passage showed similar expression profile between the different methods. These findings indicate that our novel method can generate high-quality iPSCs by direct reprogramming route with increased reprogramming speed and self-renewal capability (Sokka, Yoshihara et al. *submitted*).

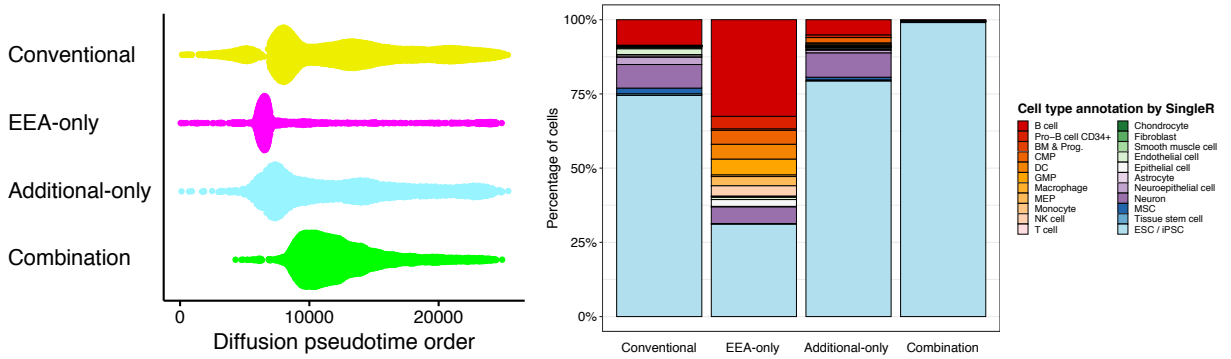

Figure 2. Characterization of cells during the reprogramming process with different methods at single-cell level. (Left) Diffusion map pseudotime analysis showing the predicted order of the samples. (Right) Cell type annotation. Conventional: conventional transgenic method, EEA-only: CRISPRa targeting OSKML with EEA-motif, Additional-only: CRISPRa targeting OSKML with the specific region, Combination: CRISPRa targeting OSKML, EEA-motif, and the specific region.

III. Transcriptional reprogramming of human embryonic stem cells to blastomere-like cells

Our group investigated the dynamics of the human preimplantation transcriptome and identified several transcription factors that are upregulated during the early embryogenesis (Töhönen et al. *Nat Commun*. 2015). These observations prompted us to test whether these transcription factors can reprogram the cells into an early embryonic-like state, earlier than blastocyst where embryonic stem cells (ESCs) are derived from. However, unexpectedly, overexpression of these genes were too unphysiological for somatic cells in many cases. Therefore we changed our initial plan and aimed to convert the human ESCs (hESCs) to blastomere-like cells. I designed and started this project, and identified the condition where the EGA genes could be activated with little cytotoxicity.

We performed time-series single-cell RNA-seq (scRNA-seq) on the hESCs collected at 6 h, 12 h, 24 h, and 48 h after induction and without induction, and I discovered that over 6% of cells collected at 12 h after induction showed similar expression profile with 8-cell stage cells (**Figure 3**). Interestingly, these cells showed upregulation of EGA genes and downregulation of pluripotency marker genes as observed in mouse 2-cell-like cells (2CLCs; Macfarlan et al. *Nature*, 2012) which has never been reported in human. I further found that a subpopulation of cells collected at 24/48 h after induction showed a high signature of naïve pluripotent stem cells, which is known to represent a relatively earlier developmental stage than primed pluripotent stem cells, suggesting that reprogrammed cells can differentiate. Finally, I identified a cell surface marker that is highly expressed in 8-cell stage cells and succeeded in the enrichment of the reprogrammed cells by fluorescence-activated cell sorting (FACS) with an antibody against the extracellular domain of this protein. These cells can become a powerful tool to model transcriptional dynamics and regulation during early human embryogenesis (Yoshihara[#] et al. *submitted*).

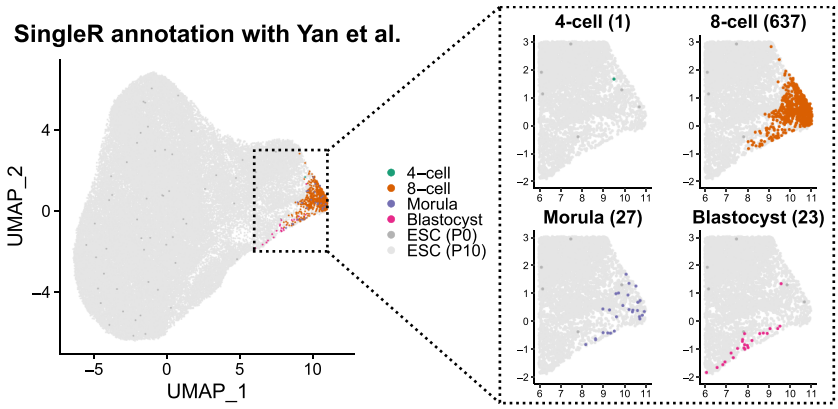

Figure 3. Cell type annotation with human preimplantation embryos and hESCs. The right four panels show the magnified plots of the cells annotated as early embryonic stage cells. Numbers in parentheses indicate the number of the annotated cells. P0: passage 0, P10: passage 10.

IV. Gene expression profiling of neuronal cell differentiation using cell models

Because the regulatory mechanism of neuronal differentiation is still largely unknown, we set up several models derived from iPSCs or embryo for studying neuronal differentiation, and investigated transcriptional changes during the differentiation.

i) iPSC-derived neuroepithelial stem cells (NES)

Developmental dyslexia is a neurodevelopmental condition with complex genetic mechanisms. A number of candidate genes have been identified, however, expression and regulation of these genes in human brain development and neuronal differentiation remain uncharted (Kere, *Biochem Biophys Res Commun.* 2014). Here I characterized the gene expression changes during the differentiation process of NES derived from iPSCs (**Figure 4-1**), and found that genes related to cilia are significantly enriched among the upregulated genes. I also found several candidate genes of developmental dyslexia are also upregulated during the differentiation. These results suggest a role of ciliary genes in neuronal differentiation and show that NES cells can provide a relevant human neuronal model to study developmental dyslexia (Bieder*, Yoshihara* et al. *Mol Neurobiol*. 2020)

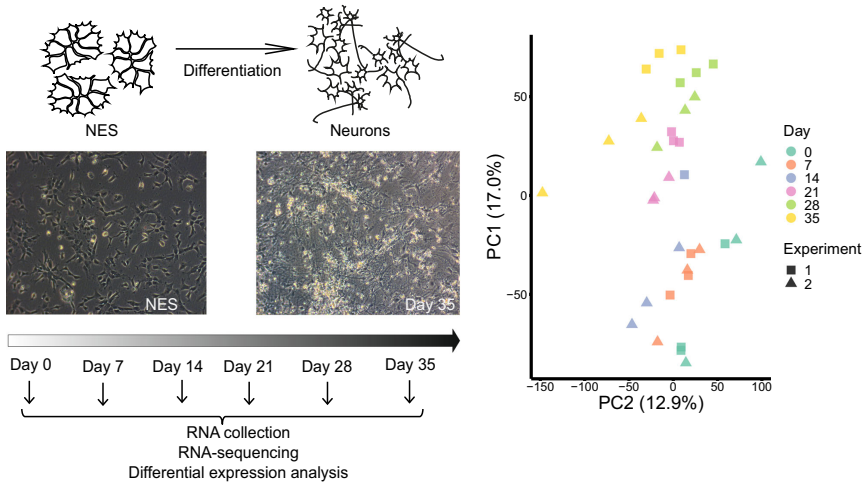

Figure 4-1. Study design (left) and principal component analysis (right) of the neuronal differentiation model.

ii) Lund human mesencephalic (LUHMES) cell line

I also characterized the gene expression changes during the differentiation process of LUHMES cell line, derived from human fetal mesencephalon. Here I found again that ciliary genes are upregulated during differentiation, and also discovered that the binding motif activity of RFX transcription factors, which are known to be key ciliogenic regulators, are drastically upregulated during the differentiation (**Figure 4-2**). I further demonstrated that gene expression dynamics of LUHMES differentiation mimic that of human fetal midbrain *in vivo*, using the publicly available single cell RNA-seq data (La Manno et al. *Cell*. 2016). These findings indicate that LUHMES is a good human neuronal cell model to study cilia for the neuronal development (Lauter*, Coschiera*, Yoshihara* et al. *J Cell Sci*. 2020).

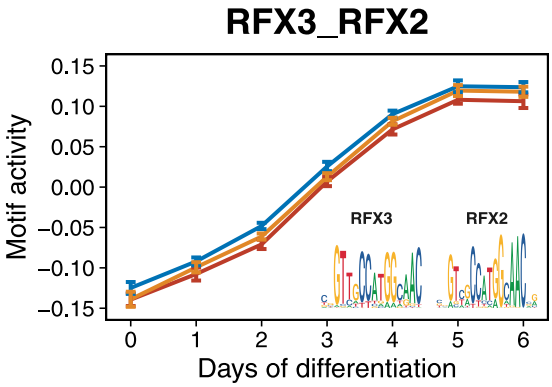

Figure 4-2. RFX3 and RFX2 binding motif activity changes during LUHMES differentiation into neurons. Different line colors represent independent sequencing libraries analyzed.

iii) Neural progenitor cells derived from cystatin B (CSTB)-deficient embryonic mouse brain

Cystatin B (CSTB) is an endogenous cysteine protease inhibitor and known to be a causative gene for progressive myoclonus epilepsy EPM1 (Unverricht-Lundborg disease). However, its function has been poorly characterized (Pennacchio et al. *Science*. 1996). Cstb-knockout mice recapitulate key features of EPM1 including myoclonus, ataxia and progressive brain degeneration. In this study, I performed transcriptome analysis of neural progenitor cells derived from wild type and Cstb-knockout embryonic mouse brain during the differentiation process. Here I identified several differentially expressed genes between them, many of which are known to be involved in neuroinflammation. Furthermore, I found genes associated with mitochondrial respiration were significantly enriched in the downregulated genes in Cstb-knockout (**Figure 4-3**). We confirmed that the oxygen consumption rate decreased in Cstb-knockout, suggesting the mitochondrial disfunction by Cstb deficiency (Daura, Tegelberg, Yoshihara et al. *Neurobiol Dis*. 2021).

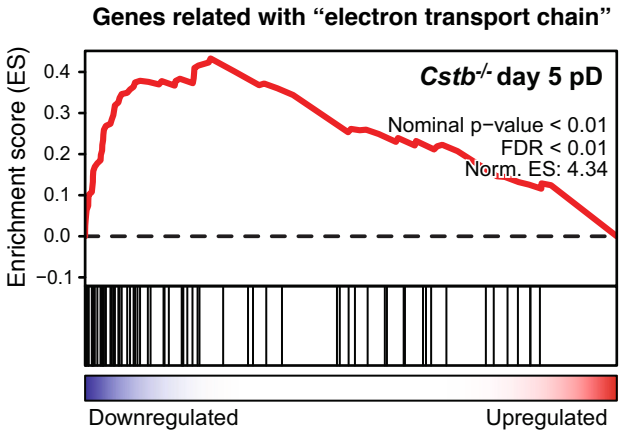

Figure 4-3. Gene set enrichment analysis of the differentially expressed genes between wild type and Cstb-knockout at day 5 post-differentiation for the GO term "electron transport chain".

V. Single-cell analysis of human ovarian cortex to investigate the existence of oogonial stem cells

There have been several reports suggesting that adult ovaries contain oogonial stem cells (OSCs) which are capable of differentiating into oocytes (White et al. *Nat Med.* 2012). If such stem cells can be isolated and proliferated *in vitro*, this would provide a new approach for infertility treatments for young women receiving cancer therapies such as chemotherapies. However, the existence of OSCs has been questioned by many researchers including our group (Zhang et al. *Nat Med.* 2015).

In this study, we performed single cell RNA-seq on healthy human ovarian cortex cells and identified six main cell types; oocytes, granulosa cells, immune cells, endothelial cells, perivascular cells, and stromal cells. I further integrated these data with the single cell RNA-seq data of human fetal ovaries (Li et al. *Cell Stem Cell.* 2017), and discovered that none of these adult cells clustered with meiotic fetal germ cells (FGCs; **Figure 5**). Although a small number of adult cells clustered together with mitotic FGCs, none of them expressed pluripotency markers, germline

markers, or oocyte markers. There results suggest that human adult ovaries do not harbor OSCs (Wagner, Yoshihara et al. *Nat Commun*. 2020). This paper was featured in some news media (The Scientist Magazine. 'Single-Cell Analysis of Ovarian Cortex Fails to Find Stem Cells' https://www.the-scientist.com/news-opinion/single-cell-analysis-of-ovarian-cortex-fails-to-find-stem-cells-67232).

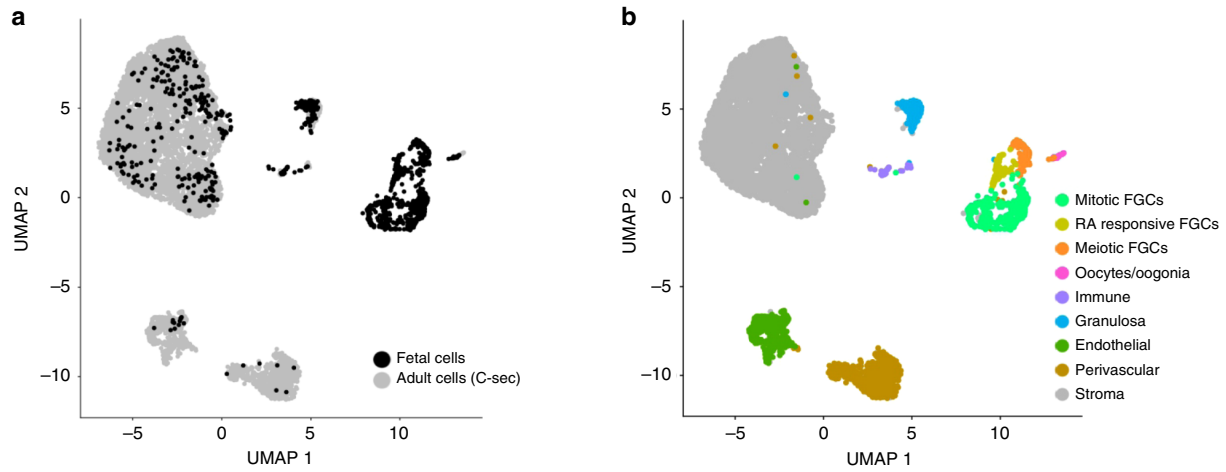

Figure 5. (a) UMAP plot showing sample origin of fetal (black) and adult cells (C-sec data, grey). **(b)** UMAP plot showing nine clusters representing different cell types. The same clusters as in adult tissue were found, as well as three new ones: mitotic fetal germ cells (FGCs), retinoic acid responsive FGCs, and meiotic FGCs.

VI. Targeted gene expression analysis of human preeclampsia placentas

As a medical doctor, I have been interested in the development of novel diagnostic methods or treatments by using transcriptome technologies. Preeclampsia is a serious pregnancy complication that threatens the wellbeing of both mother and fetus, which can cause the prematurity or even death. Here we utilized human placenta tissue samples derived from normal and severe preeclamptic donors and performed TAC-seq (Targeted Allele Counting by sequencing; Teder et al. *NPJ Genom Med.* 2018). I found *HLA-G*, regulating the immunotolerance against fetus, and its receptors (*LILRB1* and *LILRB2*) are downregulated and *IFNA1* (interferon alpha 1) is most highly upregulated in severe preeclamptic placentas (**Figure 6**) (Wedenoja, Yoshihara et al. *EBioMedicine*. 2020). These results suggest that loss of immunotolerance against fetus lead to the onset of preeclampsia, and hydroxychloroquine which is known to reduce the IFNα production might be effective as a treatment. As a matter of fact, it has been recently reported that hydroxychloroquine-treated SLE (systemic lupus erythematosus) patients showed lower risk of preeclampsia (Seo et al. *Lupus*. 2019). We are now planning a clinical trial of hydroxychloroquine treatment against preeclampsia.

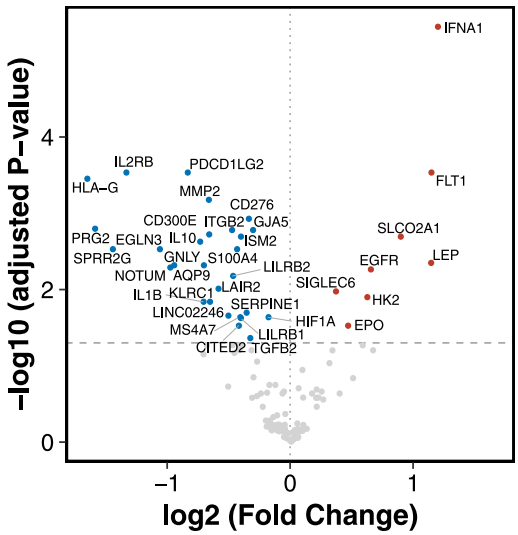

Figure 6. Volcano plot of downregulated (blue dots) and upregulated (red dots) genes (adjusted $P < 0.05$) in placentas with severe preeclampsia (n=81) versus controls (n=63).